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Glutaminases in slowly proliferating gastroenteropancreatic neuroendocrine neoplasms/tumors (GEP-NETs): Selective overexpression of mRNA coding for the KGA isoform

Monika Szeliga [a,*], Jarosław Ćwikła [b], Marta Obara-Michlewska [a], Andrzej Cichocki [c], Jan Albrecht [a]

[a] Department of Neurotoxicology, Mossakowski Medical Research Centre, Polish Academy of Sciences, 5 Pawińskiego Str., 02-106 Warsaw, Poland
[b] Department of Radiology, Faculty of Medical Sciences, University of Warmia and Mazury, 30 Warszawska Str., 10-082 Olsztyn, Poland
[c] Department of Surgical Oncology, Maria Skłodowska-Curie Memorial Cancer Center, Institute of Oncology, 15B Wawelska Str., 02-034 Warsaw, Poland

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ABSTRACT

Glutamine (Gln) is a crucial metabolite in cancer cells of different origin, and the expression and activity of different isoforms of the Gln-degrading enzyme, glutaminase (GA), have variable implications for tumor growth and metabolism. Human glutaminases are encoded by two genes: the *GLS* gene encodes the kidney-type glutaminases, KGA and GAC, while the *GLS2* gene encodes the liver-type glutaminases, GAB and LGA. Recent studies suggest that the GAC isoform and thus high GAC/KGA ratio, are characteristic of highly proliferating tumors, while GLS2 proteins have an inhibitory effect on tumor growth. Here we analyzed the expression levels of distinct GA transcripts in 7 gastroenteropancreatic neuroendocrine tumors (GEP-NETs) with low proliferation index and 7 non-neoplastic tissues. GEP-NETs overexpressed KGA, while GAC, which was the most abundant isoform, was not different from control. The expression of the *GLS2* gene showed tendency towards elevation in GEP-NETs compared to control. Collectively, the expression pattern of GA isoforms conforms to the low proliferative capacity of GEP-NETs encompassed in this study.

1. Introduction

Neuroendocrine tumors (NETs) which originate from neuroendocrine cells can arise in most organs of the body. Based on the proliferative rate, NETs are classified into well differentiated, slowly proliferating (G1 and G2 grades) and poorly differentiated, rapidly proliferating (G3 grade) (Bosman et al., 2010; Klimstra et al., 2010). The annual incidence of NETs ranges from 2 to 5 cases per 100,000 population (Yao et al., 2008; Korse et al., 2013; Scherübl et al., 2013). Gastroenteropancreatic NETs (GEP-NETs) are the most common among NETs (Verbeek et al., 2015). Treatment options for GEP-NETs consist of surgery or cytoreduction, chemo- and radiotherapy and targeted therapy and somatostatin analogs (Verbeek et al., 2015).

Glutamine (Gln) plays a crucial role in cancer cells of different origin (Wise and Thompson, 2010; Shanware et al., 2011). It is one of the major substrates for respiration and a source of precursors for the production of macromolecules. Moreover, Gln metabolism is involved in oxidative and oncogenic stress. Therefore, the expression and activity of glutaminase (GA, EC 3.5.1.2), the enzyme degrading Gln to glutamate (Glu) and ammonia, have implications in tumor growth and metabolism.

Human glutaminases are encoded by two genes: the *GLS* gene encodes the kidney-type glutaminase (KGA) which is expressed in all the tissues except liver, particularly strongly in kidney and brain (Curthoys and Watford, 1995; Aledo et al., 2000). Its alternatively spliced variant, GAC, was found in human pancreas, cardiac muscle, kidney, placenta (Elgadi et al., 1999), rat kidney, and pig renal cells (Porter et al., 2002). The *GLS2* gene encodes the liver-type glutaminase (LGA) and an alternatively spliced variant named GAB Gomez-Fabre et al., 2000; de la Rosa et al., 2009; Martin-Ruffian et al., 2012). *GLS2* is expressed in liver, brain and pancreas (Gomez-Fabre et al., 2000).

A growing body of evidence shows altered expression and/or activity of GA isoforms in different neoplastic cells and tissues. Increased activity of GA as a whole has been noted in hepatocellular carcinoma—HCC (Matsuno and Goto, 1992; Bode and Souba, 1994) and in Ehrlich Ascites Tumor Cells (Aledo et al., 1994). Co-expression of two or three GA isoforms has been revealed in human hepatoblastoma cells, breast cancer cells and myeloid cells (Perez-Gomez et al., 2005) as well as in colorectal tumor cells (Turner and McGivan, 2003). *KGA*, *GAC* and *GLS2* mRNAs are expressed in cerebral tumors (Szeliga et al., 2008), although *GLS2* is hardly detectable in highly malignant gliomas (Szeliga et al., 2005). Decreased expression of *GLS2* was also found in human hepatocellular carcinoma (Liu et al., 2014) and colon cancer tissues (Zhang

Abbreviations: NETs, neuroendocrine tumors; GEP-NETs, gastroenteropancreatic NETs; Gln, glutamine; Glu, glutamate; GA, glutaminase; KGA, kidney-type glutaminase; GAC, glutaminase C; GAB, long transcript of liver-type glutaminase; LGA, short transcript of liver-type glutaminase.

* Corresponding author.
E-mail address: mszeliga@imdik.pan.pl (M. Szeliga).

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et al., 2013). The amount of a particular GA transcript may vary depending upon cell type. The cell biological implications of co-expression of distinct GA isoforms in one and the same cell or tissue remain to be clarified. Recent studies suggest that the relative expression levels of the different isoforms may be a marker and perhaps a carrier of their proliferative capacity. In general, GLS proteins promote cancer cell growth and proliferation, whereas GLS2 proteins have an inhibitory effect on these events, which may be related to their involvement in intracellular events other than glutamine degradation, such as modulatory interactions with other proteins and/or regulation of transcription (Márquez et al., 2006; Szeliga et al., 2014; Martín-Rufián et al., 2014; Campos-Sandoval et al., 2015). With regard to GLS proteins, increasing evidence suggests that relatively high GAC and/or increased GAC/KGA ratio bespeaks high proliferation rate, as seen in gliomas (Szeliga et al., 2005) and non-small cell lung cancer (van den Heuvel et al., 2012).

So far, the expression pattern of GA isoforms in gastroenteropancreatic neuroendocrine neoplasms/tumors (GEP-NETs) has escaped any attention. In the present study, we analyzed the expression levels of distinct GA isoforms in GEP-NETs and non-neoplastic tissues. Because of limited availability of GAP-NETs, this study was confined to the low proliferating G1 and G2 tumors.

2. Materials and methods

2.1. Patients/biopsy material

Seven samples were collected from adults patients (≥18 years) affected by sporadic GEP-NETs. Six patients had functioning NETs with carcinoid syndrome located in small intestine and one patient had a non-functional pancreatic tumor. The samples were obtained during surgical resections (n = 3) or biopsy (n = 4) and immediately frozen. The tumor specimens were routinely fixed in 10% buffered formalin and embedded in paraffin. Slides were stained with hematoxylin and eosin (HE) for conventional histologic examination. The tumor cell proliferative activity was determined using Ki-67 immunohistochemistry (clone MIB-1; Dako). At least 500 tumor cells were counted to determine the percentage of cells that were positive for Ki-67. The tumors were classified into G1 (<3% positive cells) or G2 (3–20% positive cells) grades according to the WHO 2010 classification and the NANETS guidelines (Klimstra et al., 2010; Anthony et al., 2010).

The control group consisted of 7 patients who had pancreatic surgery due to the presence of pathological mass, confirmed in imaging, but without signs and symptoms which are characteristic of NEN/NETs. There were 5 patients with adenocarcinoma of the pancreas, while two other patients had surgery due to inflammatory tumor accompanying chronic pancreatitis, with no neoplastic tissue found in pathology.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2.2. RNA isolation and RT-PCR

Total RNA from frozen tissues was extracted using a guanidinium-thiocyanate-based commercial kit (TRI-Reagent, Sigma). 2 μg of RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. A volume of 2 μl of cDNA was used as a substrate for PCR. The cDNA fragments of GLS2, KGA, GAC and β-actin (constitutively expressed internal control) were amplified as described previously (Szeliga et al., 2008). PCR products were run on a 1.5% agarose gel and visualized using ethidium bromide dye. A negative control containing 2 μl of RT-omitted reaction mixture instead of cDNA did not yield any product (results not shown).

2.3. Statistical analysis

Each PCR was repeated at least 3 times on at least 2 independent cDNAs. Relative intensities of PCR products corresponding to KGA, GAC and GLS2 mRNAs versus β-actin were compared. Statistical significance of the differences was analyzed with the Mann–Whitney *U* test and the significance level was set at <0.05.

3. Results

3.1. GEP-NET patients' characteristics

The clinicopathological features are summarized in Table 1. All patients had clinical stage (CS) IV based on recent pTNM classification and ENETS recommendation (Plöckinger et al., 2004; Klöppel et al., 2007) and metastatic disease at initial diagnosis. In two cases, the Ki-67 index was <3% leading to a diagnosis of G1 and five cases, with the Ki-67 index between 3%–10% were classified as G2. During clinical follow-up 3 patients died due to GEP-NETs related causes, with the median overall survival of 72 months. Four patients are still alive, with the median overall survival of 132 months. There was no significant difference between both groups in the Ki-67 labeling index: the median value was 3.5 in alive subjects and 4.0 in dead patients.

3.2. Expression of GA isoforms in NETs and non-neoplastic samples

We have examined expression patterns of KGA, GAC and GLS2 mRNAs in 7 non-neoplastic pancreatic tissues and 7 cases of GEP-NETs. GEP-NETs presented significantly higher expression level of KGA transcript as compared to the control subjects ($p < 0.05$) (Fig. 1A). This transcript was found in all 7 tumor samples examined, but only in 4 out of 7 control tissues. Its expression level varied from 0.2 to 1.55 (median expression value: 0.4642) in tumors and from 0.0 to 1.19 (median expression value: 0.08691) in the non-tumorigenic samples. In the group of 4 subjects who are still alive, the level of KGA mRNA varied from 0.36 to 1.46 with median value 0.4485. In those patients who died, the level of this transcript varied from 0.20 to 1.61 with median value 0.9067 (Fig. 1B). The difference in the level of KGA mRNA between these two groups of patients was not statistically significant ($p = 0.6437$).

GAC was the most abundant of all GA isoforms both in GEP-NETs and controls, as its relative expression level varied from 0.99 to 3.36 (median expression value: 1.969) in tumor tissues and from 0.91 to 3.08 (median expression value: 1.814) in the controls (Fig. 2A). The difference in GAC mRNA level between GEP-NETs and control samples was not statistically significant ($p = 0.62$). No significant difference was also found when the level of GAC transcript was compared between the patients who died (median value: 1.969) and those who are still alive (median value: 1.968) (Fig. 2B).

Table 1
Patients' characteristics.

Characteristics	Data
Gender M/F	2/5
Median age at initial diagnosis (range)	55 (33–64)
Originating site: small intestine	6
Pancreas	1
WHO 2010: G1	2
G2	5
Alive/dead patients	4/3
Median overall survival (months): alive patients	132
Dead patients	72
Mean Ki-67 index: in alive patients	4.25
In dead patients	5.33

Epidemiological and clinical characteristics of patients with GEP-NET.